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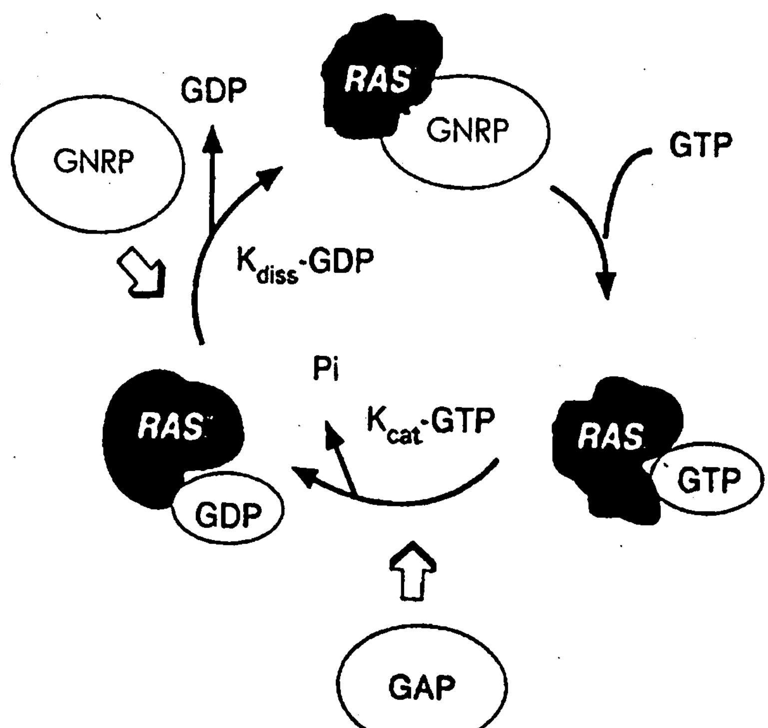
(54) Title: MUTANTS OF GNRPs AND VECTORS SUITABLE FOR THEIR EXPRESSION

(57) Abstract

The present invention relates to an amino acid sequence of a Guanine Nucleotide Releasing Protein (GNRP) spanning a portion of the catalytic domain in which threonine (T) corresponding to position 1184 of the protein of the GNRP class named $CDC25^{Mm}$ is mutated to an acidic amino acid, the gene sequence encoding said amino acid sequence, and their application in the pharmaceutical field, in particular in the treatment of tumors, cardiovascular diseases, arterial restenosis and inflammatory states, or in the diagnostic field.



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MUTANTS OF GNRPs AND VECTORS SUITABLE FOR THEIR EXPRESSION

Technical field

The technical field of the invention is the generation of mutants able to block the Ras protein signalling pathway and useful in the treatment of pathologies characterized by a functional alteration (such as hyperactivation) of Ras. In particular, the mutants of the invention are useful in the treatment of tumors, cardiovascular diseases, arterial restenosis and inflammatory states, or in the diagnostic field.

Description

The present invention concerns a mutant of a Guanine Nucleotide Release Protein (GNRP, also called GEF) spanning a portion of the catalytic domain in which threonine (T) corresponding to position 1184 of the protein called $CDC25^{Mm}$ (Swiss-Prot Accession Number P27671) is mutated to an acidic amino acid (i.e. aspartic acid or glutamic acid).

Another aspect of the invention concerns the gene sequence encoding said amino acid sequence, in which the codon corresponding to said threonine is mutated to an acidic amino acid.

The polypeptides/proteins of the invention are useful because they take part in the activation cycle of proteins of the Ras family provoking an attenuation by "sequestering" them in the form of a complex mutant GNRP/nucleotide-free Ras, so blocking the signal transduction pathway in which said Ras proteins take part; this inhibitory action on the Ras cycle has applications both in research and in the treatment of

pathologies related to functional alterations of the Ras protein.

Background Art

It is known the pivotal role played by Ras proteins in the control of cell differentiation and cell proliferation.

In their action, they act as molecular switches cycling between an active GTP·bound state and an inactive GDP·bound state, because of a transit among a series of different conformational states. Following extracellular stimuli, the inactive GDP·bound-Ras protein, releases GDP attaining a transient "empty" state, which afterwards binds GTP thus reaching the active state. The intrinsic GTPase activity hydrolyzes GTP bringing Ras back to the inactive state. This cycle is unidirectional because the GTPase reaction is irreversible and because the intracellular GTP concentration is about 10 times higher than GDP concentration, so that is GTP that preferentially binds the "empty" state.

The above described Ras cycle, and thus the levels of active Ras protein, depend on the relative activities of two protein classes which are the targets of extracellular signals. " GTPase Activating Proteins" (GAP) stimulate intrinsic GTPase activity of Ras proteins while the GNRPs catalyze the GDP/GTP exchange thus favoring the formation of the active Ras·GTP complex (see Fig. 1).

A series of experiments using both deletion and site-directed mutagenesis have identified some regions of interaction between the Ras proteins and the

catalytic domain of exchange factors. In particular, regions within the Loop4/switch2 (amino acids 64-77) appear important. The existence of a Ras/GNRP intermediate has been shown both *in vivo* and *in vitro* and underlies the dominant negative effect played by Ras proteins mutated in position 15, 16 and 17, which have a reduced affinity for nucleotides and sequester GNRP in the form of inactive $p21^{Ras}$/GNRP complexes.(For a recent review, see Polakis, M. and McCormick, F. (1993) J. Biol. Chem. 268, 9157-9169). More recently, the complex between the human $p21^{Ras}$ and the catalytic domain of the human GNRP hSos1 has been isolated (Boriack-Sjodin, P.A. et al., 1998 *Nature*, **394**, 337-341).

The first Ras-specific exchange factor to be cloned and sequenced was the product of the CDC25 gene of *Saccharomyces cerevisiae* (Camonis et al., *EMBO J* **5**, 375-380 1986; Martegani et al., 1986 *EMBO J* **5**, 2363-2369).

Two classes of Ras-GNRP have been so far identified in mammals: the p140 encoded by $CDC25^{Mm}$ (also called Ras-GRF) (Martegani et al., 1992 *EMBO J* **11**, 2151-57; Shou et al., 1992 *Nature* **358**, 351-354)) and mammalian Sos (Botwell et al., 1992 Proc. Natl. Acad. Sci. USA **89**, 6511-6515; Chardin, P. et al., 1993 *Science* **260**, 1338-1343). $CDC25^{Mm}$ has been the first mammalian exchange factor cloned by using functional complementation of a *cdc25* yeast mutation (Martegani et al., 1992, *supra*). The complete cDNA encodes a 140 kDa protein expressed only in the central nervous system. Highly homologous proteins have been later identified in rat brain (Ras-GRF) (Shou et al., 1992 *supra*) and in human brain (human CDC25) (Park W. et al., 1994 *Gene* **151**, 279-284;

WO93/21314). This protein contains in its C-terminal region a ca. 240 amino acid domain structurally and functionally homologous to the catalytic region of yeast CDC25 (Figure 2). Both the full length 140 kDa protein and truncated forms spanning C-terminal regions are active in yeast where they can substitute for endogenous CDC25, moreover they are efficient exchange factors *in vitro* both for human $p21^{Ras}$ and yeast RAS2, while they are inactive on other Ras-like proteins (Ral; Rap, Rac etc.). Both the full length p140 and the truncated forms are efficient activators of $p21^{Ras}$ *in vivo* and potentially transforming (Zippel et al., 1994 International J. Oncology 4, 175-179; Cen, H. et al., 1993 Mol. Cell. Biol. 13, 7718-7724).

The catalytic domains of $CDC25^{Mm}$-like and Sos-like GNRP are extremely conserved with each other and with *S. cerevisiae* CDC25 both from a structural and functional point of view as shown by the ability of said mammalian GNRP catalytic domains to complement *cdc25* mutation in *S. cerevisiae*.

Ras proteins, once switched to the active state in the GTP·bound form may interact *via* the L2 region with their target or effector. This leads to cascade activation of the "Mitogen Activated protein Kinases" (MAPK) or "Extracellular signal Regulated Kinases" (ERK) (Marshall CJ, 1995, *Cell* 80, 179-185; Burgering BMT and Bos JL, 1995, *TIBS* 20, 18-22). MAPK activated by dual threonine and tyrosine phosphorylation migrates in the nucleus where it can phosphorylate transcription factors inducing transcription of several genes, such as *fos*.

Summary of the invention

The amino acid threonine, in position corresponding to amino acid 1184 in $CDC25^{Mm}$ (we will refer to the full length protein unless otherwise indicated by a different subscript) is highly conserved in the catalytic domain of GNRP. It has now been found that mutation of such amino acid with an acidic amino acid, preferably with glutamic acid, is able to " down-regulate" the Ras-mediated signal transduction pathway, by binding to Ras proteins in a stable manner, thereby " sequestering " them in the form of an inactive Ras•GNRP complex.

Such mutation has been shown to be dominant-negative, because of the fact that a GNRP protein with a mutation corresponding to T1184E of $CDC25^{Mm}$ can associate irreversibly to Ras. The finding that mutant GNRP molecules - comprising in the term " molecule" entire proteins or their peptidic fragments - display such effect, allows to put in practice targeted pharmacological interventions in pathological alterations in which the Ras pathway is activated, such as neoplastic growth or neointima formation following angioplastic surgery.

Detailed description of the invention

The amino acid threonine, in position corresponding to amino acid 1184 in $CDC25^{Mm}$ is conserved in the catalytic domain of GNRP. This residue has been substituted in $CDC25^{Mm}$ catalytic domain with glutamic acid (E). The same substitution has been made both in the wild type and in the $CDC25^{Mm}$S1124V mutant, in which serine 1124 is substituted by the amino acid valine. In the evaluation of mutation effects in the different *in vivo* or *in vitro* assays, either full length proteins or

fragments corresponding to the catalytic domain, which in CDC25^{Mm} is C-terminal while in other GNRPs - such as the Sos proteins - is in the central part, have been used.

Mutants have been obtained by conventional means, using site-directed mutagenesis followed by plasmid construction for the expression of mutant GNRP in *E. coli*, in the yeast *S. cerevisiae* and in mammalian cells.

The biological activity of different mutant CDC25^{Mm} has been assayed using the obtained constructs in experiments of complementation of the temperature sensitive growth defect of the TC7 yeast strain (*MAT a ade lys trp ura3 cdc25-1*^{ts}, Martegani *et al.*, 1986 *supra*), which is able to grow at the permissive temperature of 24° C but not at the restrictive temperature of 37° C. Transformation of the TC7 *S. cerevisiae* strain have been performed by the method of Ito (Ito *et al.*, 1983 *J Bacteriol* 153, 163-168).

Analysis of yeast complementation experiments shows that both the CDC25^{Mm}T1184E single mutant and the double mutants CDC25^{Mm}T1184E/S1124V are unable to supply the CDC25 function to the strain TC7 lacking such function at the non-permissive temperature.

A further confirmation of mutant functionality has been obtained in mammalian cells by means of a *fos*-luciferase activity assay, in which mammalian cells have been cotransfected with a plasmid expressing a mutant GNRP and a *fos*-luciferase reporter plasmid whose expression is a function of Ras activity, since it is known that Ras activation brings about transcriptional activation of cellular *fos* gene.

The Ras activation state, and the exchanger activity as well, can thus be indirectly determined by assaying the activity of the enzyme luciferase which accumulates following transcription of the luciferase gene controlled by the *fos* promoter. Overexpression of the cataytic domain of $CDC25^{Mm}$ results in a significative increase of *fos*-luciferase activity in a model system where mouse fibroblasts (NIH3T3) are cotransfected with plasmids expressing $CDC25^{Mm}$ in its full length form or in a form limited to the catalytic domain only, aminoacids 976-1262, and a reporter *fos*-luciferase plasmid in which the luciferase gene is under the control of a fragment of the promoter of the human *fos* gene (-711/ +42) (Zippel *et al.*, supra; Zippel *et al.*, 1996 *Oncogene* 12, 2697-2703).

In fibroblasts transfected with the mutants of the invention, the signal transduction pathway downstream of Ras, is strongly inhibited. In fact the levels of Ras-dependent fos-luciferase activity are strongly reduced in cells expressing mutants $CDC25^{Mm}$T1184E and $CDC25^{Mm}$T1184E/S1124V both under basal conditions - serum-starved cells - and under conditions of stimulation with platelet derived growth factor (PDGF), and in cells expressing an activated (oncogenic) $p21^{Ras}$ variant. The efficacy of the dominant negative mutants described in the invention in inhibiting Ras activity is due to the irreversible GNRP-Ras binding as shown by exchange assays in which it has been shown that mutant proteins can compete *in vitro* with "wild type" exchanger blocking its effect of induction of guanine nucleotide exchange on Ras.

In other words said mutant proteins play a "sequestering" role on Ras protein in an inactive state, being able to bind it in a non functional way, so blocking the signal transduction pathway downstream. On the basis of the competition experiments it can be suggested that the GNRP mutation allows to stabilize $p21^{Ras}$ in its empty nucleotide-free state, i.e. the mutant GNRP(s) cause(s) dissociation of the Ras·GDP complex without promoting nucleotide exchange, i.e.; GTP under normal intracellular conditions.

Moreover, in further cell culture experiments it has clearly been shown that $CDC25^{Mm}T1184E$ is able to completely inhibit fibroblast transformation due to oncogenic k-*ras* expression and to slow down tumor growth in nude mice.

The possibility to block Ras activity finds several applications in the treatment of pathologies derived from a Ras hyperactivation state.

In fact all oncogenic $p21^{Ras}$ versions present point mutations in amino acids important for the binding to the guanine nucleotide that block Ras in the active state (Ras·GTP) or make its formation easier (Lowy DR and Willumsen BM, 1993 *Ann Rev Biochem* 62, 851-891).

In a particular cell type, one *ras* mutation can predispose to a particular type of tumor: for instance in a cell of the lung epithelium can predispose to an adenocarcinoma.

Literature data have recently highlighted the applicability of Ras antagonist molecules in pathological situations different from tumors. In particular it has been shown how proliferation of VSMC

(Vascular Smooth Muscle Cells) induced by PDGF, FGF (Fibroblast Growth factor) or thrombin is associated with Ras induction and that VMSC with a dominant negative Ras mutant display a significative reduction of proliferation induced from the same growth factors (Irani et al., *Biochem Biophys Res Comm* 202, 1252-1258). The same Ras mutant has been subsequently tested *in vivo* in a rat angioplastic model and a significative inhibition (60%) of neointima formation has been obtained 14 days after surgical operation (Indolfi *et al.*, 1995 *Nature Medicine* 1, 541-545). Moreover, recent studies have shown how chemotactic chemokines directly induce Ras (Knall et al., 1996, J Biol Chem 271, 2832-2838) or molecules related to Ras intracellular activity (Bokocќ , G.M. 1995, *Blood* 86, 1649-1660) so extending the pharmacological field of action of specific Ras antagonists towards biological effects non exclusively related to proliferation, such as cell motility.

Thus the dominant negative GNRP mutants of the invention can be effectively employed not only in the oncological field, but also in cardiovascular disorders, such as arterial restenosis following angioplastic therapy, or in the treatment of inflammatory states.

It is present in literature the description of a single GNRP mutant (in the *S. cerevisiae* CDC25 gene) displaying some properties of a dominant-negative mutant because of one or more missense mutations within the catalytic domain (Park, W. *et al.*, 1997 *Oncogene* 14, 831-836). The mutated amino acid in said publication is different from the amino acid residues object of the present invention. The mutant is described from the

authors as dominant-negative on the basis of the observation that its expression slows down the rate of exponential growth in yeast. No evidences are however available that such mutant can effectively inhibit Ras-dependent proliferation in mammalian cells, and if so, to what extent.

On the basis of what said above, a first object of the invention refers to an amino acid sequence of a Guanine Nucleotide Releasing Protein (GNRP) spanning a portion of the catalytic domain in which threonine (T), corresponding to position 1184 of the GNRP protein $CDC25^{Mm}$, is mutated to an acidic amino acid (i.e. aspartic acid or glutamic acid).

Among the amino acids suitable for the mutation, the substitution with glutamic acid is preferred.

The mutant's sequence can be extended to the whole protein, to the catalytic domain or, more generally, to whatever part of the molecule, provided that a certain number of upstream and downstream amino acids with respect to mutated threonine are included, for a minimum of three amino acid upstream and three amino acid downstream of the mutated site, and provided that the peptide/protein is able to bind proteins of the Ras family competing with native GNRP proteins, preferably with dominant-negative properties.

Such sequence could also contain a further mutation in the position corresponding to position 1124 of the full length $CDC25^{Mm}$ protein wherein serine is substituted by valine. Also in this case the sequence could be down-sized to a portion of the full length protein, provided that such a fragment is able to bind to proteins of the

Ras family in competition with native GNRPs, and , besides the mutated sites, it preferably spans at least three amino acids upstream and three amino acids downstream of said sites.

It will be possible to incorporate the mutant's amino acid sequence in a fusion protein or combined in such a way to obtain chimaeric proteins with the desired pharmacological properties. It will also be possible to chemically modify peptides in order to increase their *in vivo* stability. Unless otherwise specified, the generic term " mutant" in this description and in the claims comprises both full length proteins and their fragments (i.e; the catalytic domain, indicated as $CDC25^{Mm}{}_{976-1262}$), as well as fusion proteins and chemically modified proteins above mentioned.

Another object of the invention relates to the gene sequence encoding the above described protein or peptide, in which the codon corresponding to the threonine of the catalytic domain equivalent to position 1184 in $CDC25^{Mm}$ is substituted with a codon for an acidic amino acid, preferably glutamic acid and, optionally, the codon corresponding to serine of $CDC25^{Mm}$ position 1124 is substituted with a codon for valine; vectors carrying said nucleic acid sequences, such as plasmids, RNA or DNA viruses or minichromosomes are also comprised.

The mutants of invention and their respective coding sequences can be used, as said, in the therapy of tumor forms, mainly due to Ras activating mutations, cardiovascular diseases, such as arterial restenosis or inflammatory states.

For these purposes peptides, proteins, nucleic acids or their derivatives will be delivered in adeguate pharmaceuticals compositions according to what is described, for instance, in "Remington's Pharmaceutical Sciences Handbook", Mack Publishing Company, New York USA.

Alternatively, it will be possible to deliver vectors such as plasmids, locally or, when necessary, gene therapy will be used, for instance using suitably modified viral or retroviral vectors, carrying the above described gene sequences.

The compositions according to the invention will contain an effective quantity of mutant, variable as a function of the delivery route, of the pathology to be treated, of general patient conditions and will be preferentially delivered by parenteral route, in particular by intramuscular or subcutaneous injection. Also the intravenous route can be conveniently used for their delivery, provided that it is compatible with the drug properties and the desired effects.

Of course, also the daily dosage will be affected by several factors, such as pathology severity, weight, age and sex of the patient.

Other delivery routes are also possible, such as the oral route, by using formulation of the polypeptides in liposomes or other techniques known for polypeptide or protein delivery by gastroenteric route, such as those described in WO93/25583.

The mutants of the invention, their respective coding sequences or their derivatives (vectors, chimaeras, etc.) are also useful in diagnostic or

research applications. In particular, the application provides a method for screening those compounds which are able to selectively disrupt the Ras/GNRP complex. Both *in vivo* and *in vitro* assays can thus be devised in order to screen for $p21^{Ras}$ inhibiting molecules. By using the recently described inverse two hybrid technique (Vidal, M. Brachmann, R., Fattaey, A., Harlow, E., Boecke, J.D. 1996 Proc. Natl. Acad. Sci. USA 93, 10315-10320) molecules disrupting the interaction between GNRP-mutants and Ras can be isolated by positively screening for fluoroorotic-resistant colonies. The screened molecules may include either cDNA and/or oligonucleotide libraries or (combinatorial) libraries of chemical compounds. Another technique which may be used in screening compounds disrupting Ras/GNRP interaction is Scintillation Proximity Assay, SPA (EP0154734). The *in vitro* assay may comprise: a) providing GNRP-mutants, either by themselves or as fusion proteins, whereby interaction of mutants with Ras results in an easily scorable property, b) contacting the complex with a candidate agent, c) measuring the scorable property, d) comparing the scorable property in the presence of the candidate agent to that of the untreated control. Differently, the *in vivo* assay may comprise: a) providing a cell expressing the GNRP-mutants either by themselves or as fusion proteins, whereby the expression of said mutants and/or their interaction with Ras results in an easily scorable phenotype, b) contacting the cell with a candidate agent, c) measuring the scorable phenotype, d) comparing the scorable phenotype in the presence of the candidate

agent to that of the untreated control.

Brief description of the drawings

Figure 1 shows a scheme of the Ras cycle,

Figure 2 shows a scheme of Ras-specific exchange factor of the Sos and CDC25-like family,

Figure 3 shows that the catalytic domain of $CDC25^{Mm}{}_{976-1262}$T1184E is able to dissociate non-catalytically Ras-bound nucleotide, i.e. only when present in equimolar amounts compared to the $p21^{Ras}$ protein. Adding $CDC25^{Mm}{}_{976-1262}$T1184E to samples where the exchange reaction catalyzed by $CDC25^{Mm}{}_{976-1262}$ " wild type" is taking place, results in the prompt decrease of nucleotide bound to $p21^{Ras}$, indicating that the mutant GNRP forms a stable complex with nucleotide-free $p21^{Ras}$. In the body of the drawing, high concentration indicates that GNRP is present at a 1:1 molar ratio with $p21^{Ras}$. Low concentration indicates that the GNRP is present at a 1:10 molar ratio with $p21^{Ras}$.

Figure 4 shows titration of wild-type and mutant GNRP/Ras complex with mGDP. The binary protein complex (100 nM) in 50 mM Tris-Cl pH 7.5, 10 mM $MgCl_2$, 5 mM DTE at 20° C was incubated with the indicated concentrations of mGDP (A) or GTP (B), and fluorescence emission recorded. (X) $CDC25^{Mm}{}_{976-1262}$ wild-type; (▲), $CDC25^{Mm}{}_{976-1262}$T1184E. Data were plotted after subtraction of background fluorescence.

Figure 5 shows the effect of guanine nucleotides on the dissociation of the binary complex between nucleotide-free Ras and wild-type and mutant $CDC25^{Mm}$. 100 nM of the purified nucleotide-free $p21^{Ras}$/GST-$CDC25^{Mm}{}_{976-1262}$ complex was incubated with various nucleotide

concentrations and allowed to bind to glutathione-sepharose beads. After extensive washing, the proteins bound to the beads were solubilized in sample buffer, separated by SDS-PAGE, transferred to nitrocellulose and immunoblotted for the presence of $p21^{Ras}$ and $CDC25^{Mm}_{976-1262}$. Nucleotide concentrations were as follows: lane 1: no nucleotide; lane 2: 100 nM; lane 3: 500 nM; lane 4: 1 µM; lane 5: 5 µM; lane 6: 10 µM; lane 7: 100 µM.

Figure 6 shows inhibition of the activity of a Ras-dependent reporter gene (*Fos*-luciferase) by mutant $CDC25^{Mm}$T1184E. Data are average + standard deviation of three experiments performed on quiescent cells (white bars), cells stimulated with PDGF for 16 hours before assay (striped bars) or co-transfected with an oncogenic form of Ras (RasLeu61, black bars).

The following examples will be used to clarify the invention

Example 1

Construction of mutant GNRPs by site-directed mutagenesis

For all standard recombinant DNA manipulations, conventional procedures have been used unless otherwise indicated. A complete collection of such procedures is reported for instance in Sambrook *et al.*, Molecular Cloning, 2nd edition (1989) Cold Spring Harbour Laboratory Press, Cold Spring Harbour, NY USA). The mutants of interest have been prepared both in wild type $CDC25^{Mm}$ cDNA and in the mutant $CDC25^{Mm}$S1124V. In this last mutant codon 1124 that in wild type encodes a serine has been mutagenized to valine. Any conventional method known in the art can be chosen to effect mutagenesis.

Similarly, when more then one synonymous codon exists, any of these codons can be chosen for mutagenesis. In more detail, mutagenesis of codon 1124 has been effected as follows. The DNA to be mutagenized has been cloned in an expression vector called pALTER-1 (Promega), plasmid carrying a bacteriophage DNA replication origin (M13 and R408) and two antibiotic resistance-encoding genes. One of these genes, encoding tetracyclin resistance is always functioning. The other, encoding ampicillin resistance, is instead inactive. After infection with R408 of an *E. coli* culture previously transformed with the above-mentioned construct, it is possible to obtain phage particles carrying single strand plasmid DNA (ssDNA). Mutagenesis is based on the use of two primers. One primer is able to recover the Ampicillin resistance, the other is designed with one or more mismatches necessary to introduce the desired amino acid substitution in the gene product of interest. After *in vitro* synthesis of the second DNA helix, it is transformed into an *E. coli* strain mutated in the DNA repair mechanism (BMH 71-18 *mutS*) so that it can mantain *in vivo* the mismatches introduced with the synthetic oligonucleotides. A second transformation cycle in strain JM109 allows a correct segregation of mutant and wild type plasmids ensuring a elevated proportion of plasmids with the mutated construct. The mutagenic oligonucleotide used has been the following;

5'- AG ATC ACC TCC <u>GTC</u> ATC AAC CGC AG -3' where the mutagenic codon is underlined. Mutagenesis has been checked by direct DNA sequencing using the dideoxy chain termination method. Further details can be found in the

booklet accompanying the kit. Mutant DNA has then been reintroduced in the expression plasmid for mammalian cells, pcDNA3.

In order to obtain the mutants of interest in the codon 1184, the QuickChange™ Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA USA) has been used. Such a kit allows to effect site-directed mutagenesis on double-stranded circular DNA molecules using two complementary oligonucleotides containing the mutation of interest. To effect mutagenesis of codon ACC 1184 encoding threonine into codon GAG encoding glutamic acid in $CDC25^{Mm}$ and $CDC25^{Mm}$S1124V the mammal expression constructs pcDNA3 $CDC25^{Mm}$HaTag and pcDNA3 $CDC25^{Mm}$S1124HaTag were used. In such plasmids the complete $CDC25^{Mm}$ cDNA ORF is cloned downstream of the CMV (Cytomegalovirus) promoter and spliced *in frame* to an oligonucleotide encoding the influenza haemoagglutinin epitope tag (HaTag). In such way, the proteins expressed by such a construct bring at their C-terminal end a short tail facilitating, when required, immunological recognition. To effect mutagenesis at the 1184 threonine residue the following synthetic oligonucleotides have been used:

5'-C CTG GGG ATG TAT CTC <u>GAG</u> GAC TTG GTG TTC ATC G -3'

and

5'-C GAT GAA CAC CAA GTC <u>CTC</u> GAG ATA CAT CCC CAG G -3'

each one complementary to the opposite strands to mutagenize, except for the three base " mismatch" (underlined) allowing substitution of encoded amino acid from threonine to glutamic acid. The introduction of the mutation of interest also results in the introduction of

a restriction site for the enzyme *Xho*I (CTCGAG) which does not cut in the coding sequences in the starting plasmids. The mutagenesis procedure consists in the hybridization *in vitro* of the oligonucleotide primers to the previously denatured plasmid DNA template to be mutagenized. The oligonucleotide primers are then extended by PCR using PfuTurbo DNA polymerase and from extensione of said primers a nicked mutant plasmid is generated. At the end of PCR, samples are restricted with *Dpn*I restriction endonuclease (restriction site 5'-G^{m6}ATC-3') which is specific for DNA fully or hemi-methylated and which is used to digest template parental DNA, so allowing to select *in vitro* synthesized DNA carrying the mutation of interest. This latter is then used to transform the bacterial strain XL-Blue. Further details can be found in the booklet accompanying the kit. From some transformant colonies, plasmid DNA has been extracted and *Xho*I-restricted to verify mutagenesis. Some plasmids positive to the restriction analysis have been sequenced by the enzymatic dideoxy chain termination method, thus confirming that the following expression constructs for mammalian cells have been obtained:

pcDNA3CDC25^{Mm}T1184E-HaTag

pcDNA3CDC25^{Mm}T1184E/S1124V-HaTag

Example 2

Construction of plasmids for expression of mutant GNRPs in the yeast S. cerevisiae

In order to construct plasmids for expression of the TE and TE/SV mutants of the catalytic domain (residues 976-1262) of CDC25^{Mm} in yeast, the 983 bp *Eco*NI(Klenow-

filled)-*Xba*I fragments excised from plasmids pcDNA3CDC25^{Mm}T1184E-HaTag and pcDNA3CDC25^{Mm}T1184E/S1124V-HaTag were subcloned in the pVTU vector cut with *Bam*HI (Klenow-filled) and *Xba*I and dephosphorylated (more specifically plasmids pcDNA3CDC25^{Mm}T1184E-HaTag and pcDNA3CDC25^{Mm}T1184E/S1124V-HaTag were restricted with *Eco*NI, the generated ends made flush by using the Klenow fragment of DNA polymerase I, later digested with XbaI; the excised 983 bp fragment was resolved by preparative agarose gel electrophoresis, purified from the gel and ligated with the yeast expression vector pVTU which in turn was restricted with *Bam*HI, had the generated ends made flush with the Klenow fragment, followed by digestion with *Xba*I and dephosphorylation). The constructs pVTUCDC25$^{Mm}_{976-1262}$T1184E-HaTag and pVTUCDC25$^{Mm}_{976-1262}$T1184E/S1124V-HaTag have been so obtained. Insertion of each fragment in the proper orientation was checked by retriction analysis.

Example 3

Construction of plasmids for the expression in E. coli of hybrid proteins between glutathione-S-transferase and mutant GNRPs

The starting point for the construction of plasmids expressing in *E. coli* hybrid proteins between glutathione-S-transferase (GST) and CDC25^{Mm}976-1262 was plasmid pGEX2TCDC25^{Mm}976-1262 (Martegani *et al.*, 1992 supra). Such plasmid expresses fusion proteins between GST and the catalytic domain of CDC25^{Mm} (residues 976-1262) under the control of an IPTG-inducible promoter. Plasmid pGEX2T- CDC25^{Mm}976-1262 was restricted with *Nde*I and *Eco*RI which results in excision of a 430bp fragment;

the deleted plasmid was freed of the excised fragment by preparative agarose gel electrophoresis and after dephosphorylation was ligated to the 480 bp *NdeI-EcoRI* restriction fragment excised from plasmids pcDNA3CDC25^{Mm}T1184E-HaTag and pcDNA3CDC25^{Mm}T1184E/S1124V-HaTag. The plasmids pGEX2T-CDC25$^{Mm}_{976-1262}$T1184E-HaTag and pGEX2T-CDC25$^{Mm}_{976-1262}$T1184E/S1124V-HaTag have been so obtained. Insertion of each fragment in the proper orientation was checked by restriction analyses.

Example 4

Mutant GNRP: complementation assay in S. cerevisiae cdc25 mutants

Cells transformed with plasmids described in the Example 2 were plated on minimal selective medium, with glucose as a carbon source. The transformant plates after an incubation at 24°C for 36 hours (permissive temperature) were shifted to restrictive temperature (36°C). In such conditions the mutant strain does not grow, while the mutant transformed with wild type CDC25^{Mm} gives visible colonies 48-72 hours after the shift at the restrictive temperature.

Flasks used for yeast growth in liquid medium were incubated in a Dubnoff water bath with shacking. Growth on plates was done in a humidified atmosphere incubator. For all methods regarding yeast not explicitly described, see Guthrie and Fink, *Methods in Enzimology* 194).

The ability of each mutant to complement, at the restrictive temperature of 37°C, the *cdc25-1*^{ts} mutation was scored; results reported in Table 1 give the ratio between colonies grown at 37°C and those grown at 24°C.

Average ± standard deviation from at least three independent experiments is reported.

Table 1 *Functional complementation of the S. cerevisiae cdc25 mutation by mutant GNRP*

Plasmid	Colonies (37°C/ 24°C)
pVTU	<0.002
pVTU-CDC25^{Mm}	0.90 ± 0.09
pVTU- CDC25^{Mm}T1184E	< 0.002
pVTU-CDC25^{Mm}TS1124V	< 0.002
pVTU- CDC25^{Mm}T1184E/S1124V	< 0.002

Example 5

Expression in E. coli and purification of mutant GNRP

Purification was carried out at 4°C. Purification of GST-CDC25^{Mm}$_{976-1262}$ fusion proteins is an affinity chromatography which utilizes the glutathione-sepharose resin (Sigma).

The protocol employed is very similar for the purification of wild type and mutant CDC25^{Mm}$_{976-1262}$ proteins. The major steps can be summarized as follows:

- Inoculate the *E. coli* strain transformed with the desired plasmid in LB plus ampicillin (100 μg/ml). Incubate at 37°C over/night.
- The next day 8 ml of preinoculum are diluted in 500 ml LB + ampicillin medium till the optical density at 600 nm of the bacterial culture reaches 0.4-0.6 OD.
- Protein production is induced with 0.2 mM IPTG (Isopropyl-β-D-Thiogalactopyranoside) for 16 hours at 24°C (mutants are induced with 0.05 mM IPTG for 3 hours).

- Cells are collected by centrifugation at 6000 rpm for 10 minutes.
- Dissolve cell pellet in 8 ml of lysis buffer made as follows: PBS 1x (NaCl 150 mM, Na_2HPO_4 16 mM, NaH_2PO_4 4 mM pH 7.3), β-mercaptoethanol 14 mM, EDTA 1 mM, Pefablock 0.5 mM, 0.5 % Triton X-100.
- Cells are broken at 4°C by sonication.
- 1% Triton X-100 and 50 DNase I units are added.
- Centrifuge 20 minutes at 15000 rpm at 4°C.
- Supernatant is recovered and incubated with 50% resin (washed three times with PBS 1x) for 90 minutes at 4°C with mild agitation.
- Centrifuge 2 min at 1500 rpm at 4°C and discard supernatant.
- Wash the resin twice with 10 ml PBS, 14 mM β-mercaptoethanol, 1 mM EDTA, 0.5 % Triton X-100 and twice with 50 mM TRIS-HCl pH 8.5, 50 mM NaCl, 14 mM β-mercaptoethanol, 1 mM EDTA, 0.5 % Triton X-100 (Buffer A, pH 8.5).
- Incubate the resin three times with 4 ml Buffer A pH 8.5 for 15 minutes at 4° C in the presence of 3 mg/ml reduced glutathione.
- Dialyze against 500 ml of 50 mM TRIS-Cl pH 7.5, 50 mM NaCl, 14 mM β-mercaptoethanol to concentrate ca. three times the protein.
- Check on 10 % SDS-polyacrilamide gels the different purification steps.

When required, wild type and mutant $CDC25^{Mm}$ proteins were separated from GST by thrombin cleavage as follows.

After the last washing, before elution, resin is

resuspended in Thrombin buffer (TRIS-Cl 50 mM pH 7.5, NaCl 50 mM, $CaCl_2$ 5 mM), the resin is centrifuged and resuspended in an equal volume of Thrombin buffer containing about 10 thrombin units/mg of fusion protein bound. After ca. two hours at 4°C with mild shacking, the resin is then centrifuged and the soluble fraction, containing the protein of interest, is collected. The resin is washed twice with PBS and the three soluble fractions collected are pooled and checked by SDS gel electrophoresis. A further protein purification step uses an ionic exchange colummn to eliminate thrombin residues and possible contaminating proteins. A Pharmacia MonoQ column with a 0-1M NaCl elution gradient is used; protein gets eluted at ca. 140 mM NaCl. Protein-containing fractions are controlled by SDS-PAGE and pooled, if required are concentrated with Centripep 10 (Centricon) and dialyzed over/night against 1 liter of TRIS-Cl 50 mM pH 7.5, NaCl 50 mM, glycerol 50 %, β-mercaptoethanol 7 mM.

Example 6

Guanine nucleotide exchange and dissociation assays on $p21^{Ras}$ and RAS2 proteins with GNRP mutants

The use of labeled nucleotides allows to measure dissociation rates of Ras·GDP complexes, as well as the GDP/GTP exchange reaction by means of filtration on nitrocellulose filters (Millipore, 0.45 μm). Only Ras·bound nucleotides are retained on the membrane and radioactivity determination allows to measure the amount of complex retained on the membrane. Nitrocellulose membranes, soaked in the same buffer used in the reaction, are put on a filtration apparatus connected to

a vacuum pump which allows aspiration with a pressure of 0.9 bar. After an incubation period at 30°C aliquots of the reaction are withdrawn and filtered on the membrane.

Nitrocellulose filters are later air-dried and counted in scintillation vials containing 5 ml of scintillation fluid (Ultima Gold Packard) and counted with a Prias Counter.

Dissociation reaction

Dissociation kinetics of the Ras·guanine nucleotide complexes have been studied charging p21 (or RAS2) protein with [^{3}H]GDP; dissociation of the complexes so obtained is followed as a function of time after adding an excess unlabelled nucleotide.

The p21^{Ras} or RAS2 protein (2.5 μM) is incubated in the presence of buffer A (50 mM TRIS-HCl pH 7.5, $MgCl_2$ 1 mM, 10 mM NH_4Cl, 0.5 mg/ml BSA), 3 mM EDTA and 20 μM [^{3}H]GDP.

After 10 minutes the reaction is stopped by adding 3 mM $MgCl_2$ and putting the tube on ice (Reaction 1).

Dissociation rate of the labelled complex is measured after adding a ca. 500 fold excess unlabelled nucleotide (Reaction 2).

25 μl of reaction 1 are incubated at 30°C in buffer A containing 1.6 mM unlabelled GTP (GDP) in the presence or absence of different concentrations of wild type or mutant CDC25^{Mm}. Final volume of reaction 2 is 120 μl; at predetermined times 15 μl aliquots are taken and the decrease in Ras·bound radioactivity is followed after nitrocellulose filtration as a function of time.

Exchange reaction

The exchange reaction is performed by incubating

the $p21^{Ras}$·GDP or RAS2·GDP complex in buffer A in the presence of [^{3}H]GDP (2 μM), in the presence of different concentrations of $CDC25^{Mm}$ as required. Final volume is 120 μl. The increase in Ras·bound radioactivity is followed as a function of time by nitrocellulose filtration.

The reaction is incubated at 30° C, 15 μl aliquots are taken at different time intervals and filtered on nitrocellulose; the increase in $p21^{Ras}$-bound radioactivity (filter retained) corresponds to the exchange of unlabeled GDP with labeled GTP.

EDTA at a final 3 mM concentration is used as a positive control of the exchange reaction, because by chelating Mg^{++}, it greatly accelerates the GDP/GDP exchange reaction, so giving the maximum attainable Ras·bound radioactivity at equilibrium. Under standard assay conditions (molar ratio GNRP:$p21^{Ras}$ 1:10), none of the mutants object of the invention is able to significatively stimulate exchange and dissociation of guanine nucleotides on $p21^{Ras}$.

Example 7

Induction of non-catalytic dissociation of Ras·bound nucleotides, without nucleotide exchange induction by mutant GNRP

In order to evaluate whether the mutant $CDC25^{Mm}_{976-1262}$T1184E remains bound to $p21^{Ras}$ in its empty form, we examined the effect of such mutant on the exchange and dissociation reaction at a 1:1 GNRP:$p21^{Ras}$ molar ratio. $CDC25^{Mm}_{976-1262}$T1184E is unable to promote exchange even at a 1:1 molar ratio with $p21^{Ras}$. On the contrary adding $CDC25^{Mm}_{976-1262}$T1184E provokes a rapid and dramatic drop in

$p21^{Ras}$-bound radioactivity induced by "wild type" $CDC25^{Mm}_{976-1262}$. These data indicate that, when present in equimolar ratio with Ras•GDP, $CDC25^{Mm}_{976-1262}$T1184E displays a strong dissociation action on the Ras•GDP complex, but is unable to promote exchange.

Example 8

Identification of the mechanism of action of mutant GNRP

In order to identify the mechanism of action of the dominant negative GNRP, the stability of the binary complex between the mutant catalytic domain of $CDC25^{Mm}$T1184E and nucleotide free Ras was investigated. The binary complex Ras/$CDC25^{Mm}_{976-1262}$ was prepared by incubating a 5 fold molar excess of $p21^{Ras}$•GDP in 50 mM Tris-Cl pH 7.5, 10 mM $MgCl_2$, 5 mM DTE buffer containing 10 mM EDTA with wild-type or mutant GST-$GNRP_{976-1262}$ (pre-bound to glutathione-sepharose) for 30 min at room temperature. The bound complex was washed extensively with PBS, 14 mM β-mercaptoethanol, 1 mM EDTA, 0.5 % triton X-100. The complex was eluted with reduced glutathione (3 mg/ml in 50 mM Tris-Cl pH 8.5, 50 mM NaCl, 14 mM β-mercaptoethanol, 1 mM EDTA, 0.5 % Triton X-100) dialyzed against 50 mM Tris-Cl pH 7.5, 50 mM NaCl, 14 β-mercaptoethanol, 50 % glycerol) and stored at -20°C. At least 70 % of the GST-GNRP protein was bound to $p21^{Ras}$ under these conditions as evaluated by gel electrophoresis.

<u>Effect of guanine nucleotide on dissociation of $p21^{Ras}$-GNRP complex</u> - We used mant-GDP (Fig. 4A) and mant-GTP (Fig. 4B) to investigate binding of guanine nucleotides to mutant nucleotide-free Ras/$GNRP_{976-1262}$

complexes. Guanine nucleotide carrying the mant group on ribose (mant-nucleotides) were purchased from Molecular Probes. All measurements were carried out at 20 ° C in 50 mM Tris-Cl pH 7.5, 10 mM $MgCl_2$, 5 mM DTE buffer using a Jasco FP-777 fluorometer with an excitation wavelength of 366 nm and an emission wavelength of 442 nm. Concentration as low as 25 nM of each nucleotide result in detectable specific binding of the nucleotide to the complex between nucleotide-free Ras and wild-type $GNRP_{976-1262}$. On the opposite, no specific nucleotide binding to the complexes between nucleotide-free Ras and $GNRP_{976-1262}$T1184E was detectable for nucleotide concentrations at least ten fold higher. At nucleotide concentrations higher than 1 μM the fluorescence contributed by nucleotide binding to the protein was so small in comparison to total fluorescence (less than 10 %), that no reproducible data could be obtained for concentrations exceeding this level.

<u>Dissociation of the nucleotide-free Ras/GNRP complex by guanine nucleotides</u>- In order to analyze whether reduced nucleotide binding correlates with increased stability of the binary $p21^{Ras}$/GNRP complex, the ability of guanine nucleotides to dissociate the wild-type and mutant binary complexes was directly investigated. Nucleotide dissociation of the $p21^{Ras}$/GST-$CDC25^{Mm}_{976-1262}$ complexes was assayed as follows. 100 nM of each of each purified complexes was incubated with various nucleotide concentrations in 50 mM Tris-Cl pH 7.5, 50 mM KCl, 5 mM $MgCl_2$, 5 mM β-mercaptoethanol at 4° C for 15 min and allowed to bind to glutathione-sepharose beads. After extensive washing with PBS, pH

7.4, the proteins bound to the beads were solubilized in sample buffer, separated by SDS-PAGE, transferred to nitrocellulose and immunoblotted for the presence of $p21^{Ras}$ and $CDC25^{Mm}_{976-1262}$, in order to detect association of the $p21^{Ras}$ and $CDC25^{Mm}$ proteins. Both anti-Ras and anti $CDC25^{Mm}$ antibodies were from Santa Cruz Biotechnology. Peroxidase-conjugated secondary antibodies were from Zimed. Bound antibodies were visualized with ECL (Amersham). Results are reported in Figure 5. Consistent with fluorescent experiments, these data indicate that the wild-type complex is completely dissociated by GDP concentration above 1 μM, whereas the mutant complex was barely affected by concentrations below 10 μM. The T1184E complex was only partially dissociated by 100 μM GDP. Similar results were obtained with GTP-induced dissociation of the complexes.

The major role for guanine nucleotide releasing proteins in Ras activation seems that of facilitating nucleotide release. The preferential binding of GTP over GDP appears to be due to the higher intracellular GTP concentration (Frascotti, G., Coccetti, P., Vanoni, M.A., Alberghina, L., and Martegani, E. (1991) *Biochem. Biophys. Acta* 1089, 206-212). The interaction between GNRP and Ras has to be sufficiently strong to displace the bound nucleotide and weak enough to allow entry of the nucleotide and disruption of the binary GNRP/Ras complex. The data presented in this example further support the hypothesis that the formation of the binary complex between the mutant GNRP and nucleotide-free Ras having a diminished affinity for the incoming nucleotide, would result in a stabilization of the

binary complex under conditions where the wild type complex is disrupted by the presence of nucleotides.

Example 9

Biological assay: inhibition of expression of Ras-dependent genes by mutant GNRPs in mammalian fibroblasts

In order to show *in vivo* whether proteins $CDC25^{Mm}T1184E$ and $CDC25^{Mm}T1184E/S1124V$ were able to display an inhibitory effect on Ras *in vivo*, NIH3T3 cells were transfected with plasmids expressing said proteins together with a reporter fos-luciferase plasmid whose expression is a function of ras activity since it is known that Ras activation results in the induction of transcription of the cellular *fos* gene. The Ras activation state, and hence the exchanger activity, is indirectly determined by assaying the activity of the luciferase enzyme which accumulates following the activity of the luciferase gene controlled by the *fos* promoter. Fos-luciferase activity has been assayed under basal conditions and in cells stimulated with PDGF. PDGF stimulation allows to activate Ras in a $CDC25^{Mm}$-independent way, thus reaching elevated luciferase activity values, mandatory prerequisite to show the presence of a dominant negative effect by using transient transfections (Sakaue *et al.*, *Mol Cell Biol* 15, 379-388; Zippel *et al.*, 1996 *supra*).

Luciferase activity was assayed using Promega "Luciferase Assay System®", measuring light emission with a luminometer, in conditions where light emitted in a given time interval was a linear function of added extract.

DNA used for transfections was purified by Quiagen

"Plasmid Maxi Kit®". NIH3T3 cells have been transfected using the lipofectamine® (GIBCO) technique, a liposome formulation made from the polycationic lipid DOSPA (2,3-dioleoxyl-N-(2sperminecarboxamido)ethyl)-N,N-dimethyl-1-propanium trfluoroacetate) and by the neutral lipid DOPE (dioleil phosphatydylethanolamine) in 3:1 ratio w/w with appropriate quantities of the plasmids expressing the $CDC25^{Mm}$ mutants of the invention and left for 40 hours without serum in the presence of transferrin and selenium. These factors are required, in the absence of serum, to guarantee a good cell adhesion. Luciferase activity of aliquots (10µl) of cell extracts have been measured with a luminometer and the Relative Light Units (RLU) so obtained have been normalized according to the protein content in each sample. Data have then be expressed as relative luciferase activity compared to the value reached by the same strain transfected with the control empty plasmid in any given growth condition and taken as unit, as shown in the graph in Fig. 6.

Procedure of the lipofectamine technique

$1{,}8 \times 10^5$ cells are plated in 6-wells dishes or in single 35 mm (diameter) dishes in 2 ml of growth medium (DMEM + 10 % NCS + glutamine + antibiotics) and incubated at 37 ° C in a 0.5 % CO_2 atmosphere. After 16-18 hours the DNAs are diluted at the concentration of 0.25 µg/ml with sterile dH_2O. The DNA (4.5 - 4.6 total µg per triplicate) is added to 300 µl DMEM + glutamine prewarmed at 37°C in Falcon tubes. The added DNA contains:

1. pCDNA3 + gene of interest (0.1-1.0 µg DNA)
2. pFos-Luciferase 1 µg per triplicate (0.33 µg/well)

3. pCDNA3 (empty vector) (add enough DNA to bring total DNA to 4.5-4.6 µg).

4. pRAs^{Leu61} (1.0 µg) when required.

To each tube containing the DNA mix 300 µl of lipofectamine solution - prepared by adding 18 µl of Lipofectamine to 300 µl DMEM + glutamine - are added. The solution is incubated for 30 minutes. At the end of the incubation 2.4 ml of DMEM + glutamine are added to each test tube. 950 µl of the DNA-Lipofectamine mix are added dropwise to the cells which have been washed twice with PBS 1 x. Transfected cells are incubated for 5 hours at 37° C in 0.5 % CO_2 atmosphere. At the end of the incubation, 950 µl of medium with a double serum concentration (DMEM + glutamine + NCS 20 %) are added. After 16 hours cells are washed twice with PBS to completely eliminate serum. Two ml of DMEM + NaSelenite (GIBCO 0.346 µg/ml dH_2O = 1000 x) + transferrin (4 ng/ml dH_2O = 1000 x). After about 40 hours cells are, if required, stimulated with PDGF 100 ng/ml for 16-18 hours in the presence of 0.1 % bovine serum albumin.

In order to measure the luciferase activity, medium is removed by aspiration and cells are washed twice with TBS 1 x (2 ml per well). 150 µl of reporter lysis buffer 1x (Promega) are added. Cells are incubated at room temperature for 10 minutes on a rotary shaker, then the lysate is recovered with a scraper and transferred to an Eppendorf 1.5 ml tube. Extracts are centrifuged in a minifuge at 13.000 x g for 5 minutes at 4°C, the supernatants are moved to clean eppendorf tubes and kept on ice. 50 µl of luciferase substrate are added to 10 µl of supernatant and the light produced over a 60 second

interval is measured with a luminometer. Obtained values are normalized according to the protein content of each sample.

In cells transfected with plasmids encoding the proteins $CDC25^{Mm}T1184E$ and $CDC25^{Mm}T1184E/S1124V$ the *fos* promoter is activated to levels significatively lower in comparison to cells transfected with the empty vector, both under basal conditions (Fig. 6, white bar) and in the presence of PDGF (Fig. 6, striped bars). The values reached by cells transfected with $CDC25^{Mm}S1124V$ are not statistically different from those obtained with the empty plasmid (Fig. 6). Such results thus indicate that the mutant proteins object of the invention are able to attenuate the $p21^{Ras}$-dependent signal transduction pathway.

Example 10

Biological activity assay: the mutant GNRP $CDC25^{Mm}T1184E$ and $CDC25^{Mm}T1184E/S1124V$ revert the effect of oncogenic Ras in mammalian fibroblasts

In order to further evaluate the biological effects of expression of mutants $CDC25^{Mm}T1184E$ and $CDC25^{Mm}T1184E/S1124V$ in mammalian cells, NIH3T3 cells were cotransfected, using the method described in the previous Example, in the presence of a plasmid expressing an oncogenic form of Ras, Ras^{Leu61}. The levels of fos-luciferase activity detectable under such conditions in control cells not expressing mutant GNRPs are 10 times higher than those observed in basal conditions (absence of plasmid expressing Ras^{Leu61}). The expression of the proteins $CDC25^{Mm}T1184E$ and $CDC25^{Mm}T1184E/S1124V$ results in a reduction of ca. 80 %

of such an activity. The mutant $CDC25^{Mm}$S1124V, on the contrary, has no inhibitory effect.

In further experiments, the plasmids expressing the cDNAs of the mutants of the invention and the control plasmid pcDNA3 were stably transfected in murine NIH3T3 fibroblasts transformed with oncogenic Ras and a morphological analysis of the transfected cells was performed. In fact one of the most evident effects of Ras oncogene activation is cell transformation characterized by morphological alterations, so that a possible Ras inhibition results in a regression of the transformed phenotype. Stable transfected clones with the plasmids expressing the mutants of interest and with the empty control plasmid have thus been prepared. Following transfection geneticin resistant colonies were selected (plasmid pcDNA3 carries the gene encoding resistance to such antibiotic) and isolated after about 15 days of selection. Both control colonies and the colonies transfected with the mutants object of this invention have been expanded and their morphology analyzed by optical microscopy. While control colonies displayed the typical transformed morphology, about 90 % of the colonies transfected with the mutant object of the invention presented reversion of the transformed phenotype, making these cells look the same as the parental NIH3T3 untransformed cell line.

Moreover cells transfected with plasmids expressing the mutants of the invention showed a severe delay in tumor formation upon injection in nude mice, when compared to cells transfected with the empty plasmid pcDNA3. In more detail, the ability of the above

mentioned stable transfected cells to form tumors when injected in nude mice has been assayed. About 5 x 10^4 or 10^5 cells have been subcutaneously injected in nude mice. K-ras transformed cells expressing the mutant cells object of the invention show a significative delay in tumor formation, since no tumor mass can be observed at least for a few weeks after the control tumor cells have formed the tumor.

In conclusion these results indicate that expression of mutant GNRPs object of the invention inhibits the signal transduction pathway turned on by mutated Ras, results in reversion of the phenotype of mutated k-ras-dependent tumor cells and is able to inhibit Ras-mediated tumor formation in xenotransplants.

It will be apparent to the experts in the art that the mutations T/E and S/V which form the major property of the mutants and respective encoding gene sequence of the invention (which are here exemplified by the protein $CDC25^{Mm}$), can be applied to other members of the Ras-specific GNRP family as well.

In particular, both mutants in the human and yeast homologs of $CDC25^{Mm}$ as well as mutants in human and mouse Sos proteins, fall within the scope of the invention as long as said mutants maintain properties and characteristics substantially identical to those of the illustrative $CDC25^{Mm}$T1184E and $CDC25^{Mm}$T1184E/S1124V mutants.

Claims

1. Guanine nucleotide release protein (GNRP)-mutants characterized in that they irreversibly block Ras proteins in an inactive state by forming a stable Ras/GNRP-mutant complex, said mutants characterized in that they have the amino acid threonine, corresponding to position 1184 in $CDC25^{Mm}$, mutated to an acidic amino acid.

2. GNRP-mutants according to claim 1, wherein said acidic amino acid is glutamic acid.

3. GNRP-mutants according to claim 1 and 2, characterized in that they comprise, besides the mutation site, a number of amino acids sufficient to bind Ras proteins in competition with native GNRP-proteins, said amino acids being at least three (3) amino acids upstream and three (3) amino acids downstream the mutated site.

4. GNRP-mutants according to claim 2 which correspond to mutated protein $CDC25^{Mm}$T1184E.

5. GNRP-mutants according to claim 3 which correspond to mutated $CDC25^{Mm}_{976-1262}$T1184E.

6. GNRP-mutants according to claim 1, wherein amino acid threonine corresponding to position 1184 of $CDC25^{Mm}$ is mutated into an acidic amino acid and amino acid corresponding to position 1124 of $CDC25^{Mm}$ is mutated into valine.

7. GNRP-mutants according to claim 6, wherein said acidic amino acid is glutamic acid.

8. GNRP-mutants according to claims 6-7, characterized in that they comprise, besides the mutation sites, a number of amino acids sufficient to

bind Ras proteins in competition with native GNRP-proteins, said amino acids being at least three (3) amino acids upstream and three (3) amino acids downstream the mutated site.

9. GNRP-mutants according to claim 7, which correspond to mutated $CDC25^{Mm}T1184E/S1124V$ protein.

10. GNRP-mutants according to claim 8, which correspond to mutated $CDC25^{Mm}_{976-1262}T1184E/S1124V$ protein.

11. GNRP-mutant chimaeric proteins which are obtained by combining the sequence of a GNRP-mutant according to any of claims 1-10 with the sequence of other proteins.

12. Gene sequences encoding for any of the GNRP-mutants according to claims 1-11.

13. Vectors carrying the gene sequences according to claim 12.

14. Pharmaceutical compositions containing as the active ingredient a GNRP-mutant according to claims 1-11.

15. Use of the GNRP-mutants according to claims 1-11 as a medicament.

16. Use of the GNRP-mutants according to claims 1-11 for the preparation of a medicament for the treatment of pathologies related to Ras activation.

17. Use of the GNRP-mutants according to claim 16 wherein said pathologies related to Ras activation are chosen among: tumors, cardiovascular diseases, arterial restenosis, inflammatory states.

18. Use of the GNRP-mutants according to claims 1-11 as reagents in a screening assay of compounds able to dissociate the Ras/GNRP complex.

19. Use of the gene sequences according to claim 12 and/or vectors according to claim 13 for the preparation of gene-therapy reagents for the treatment of pathologies related to Ras activation.

20. Use according to claim 19, wherein said pathologies related to Ras activation are chosen among: tumors, cardiovascular diseases, arterial restenosis, inflammatory states.

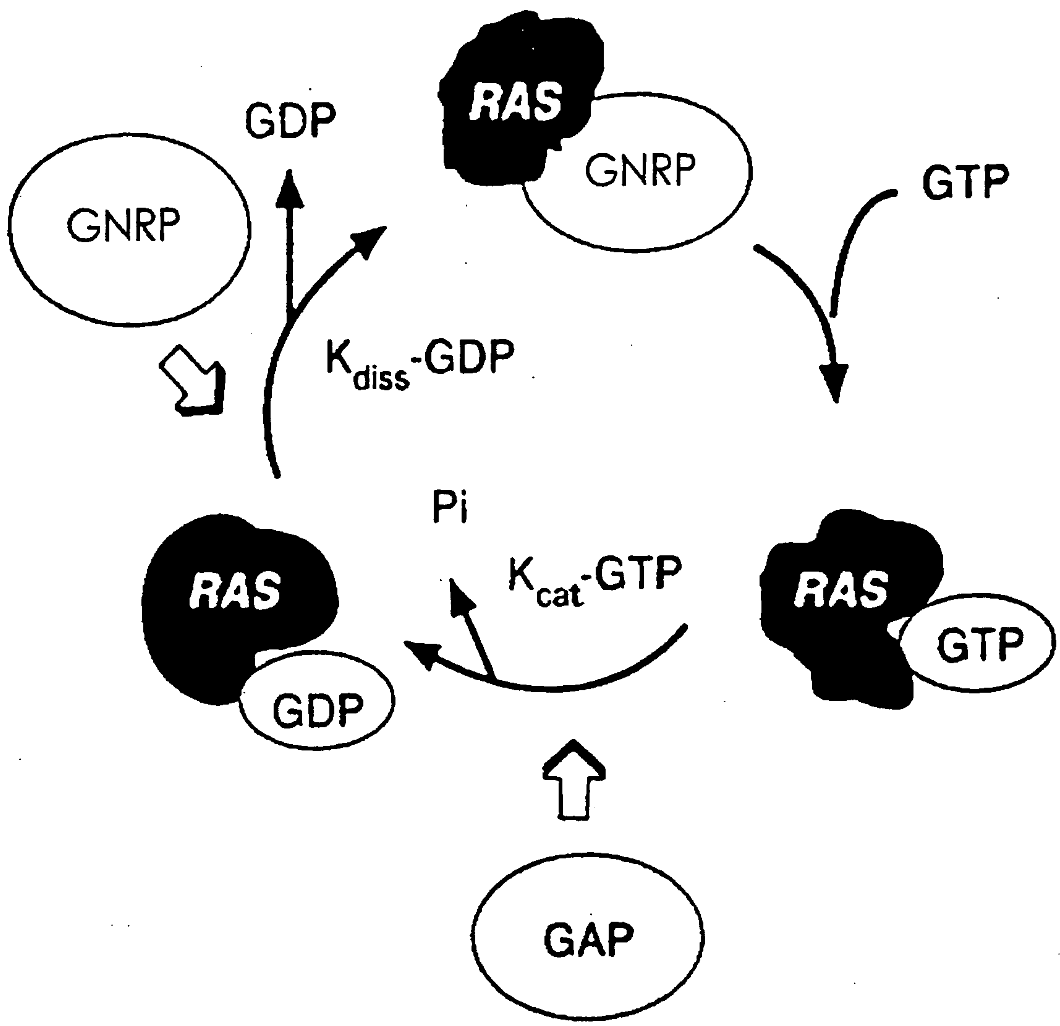


Figure 1

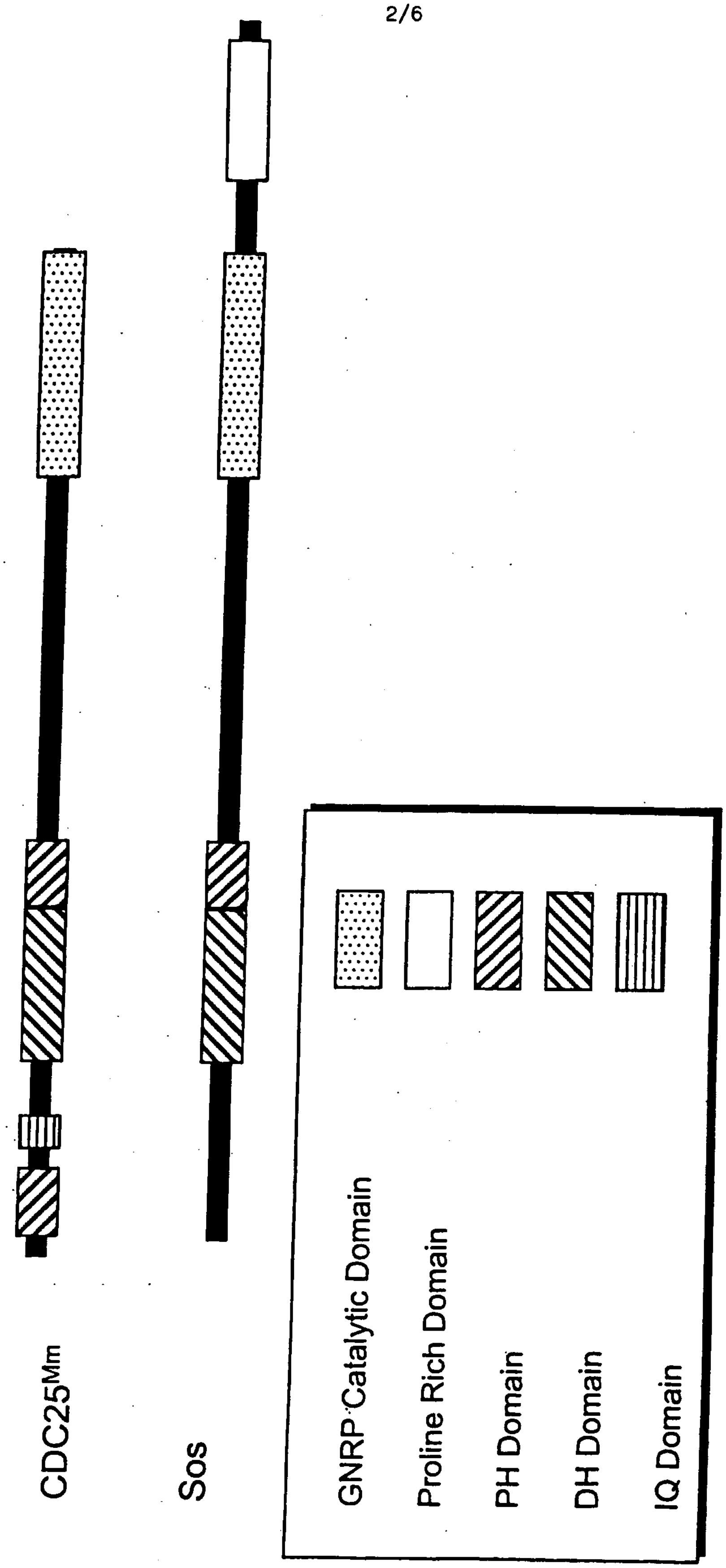
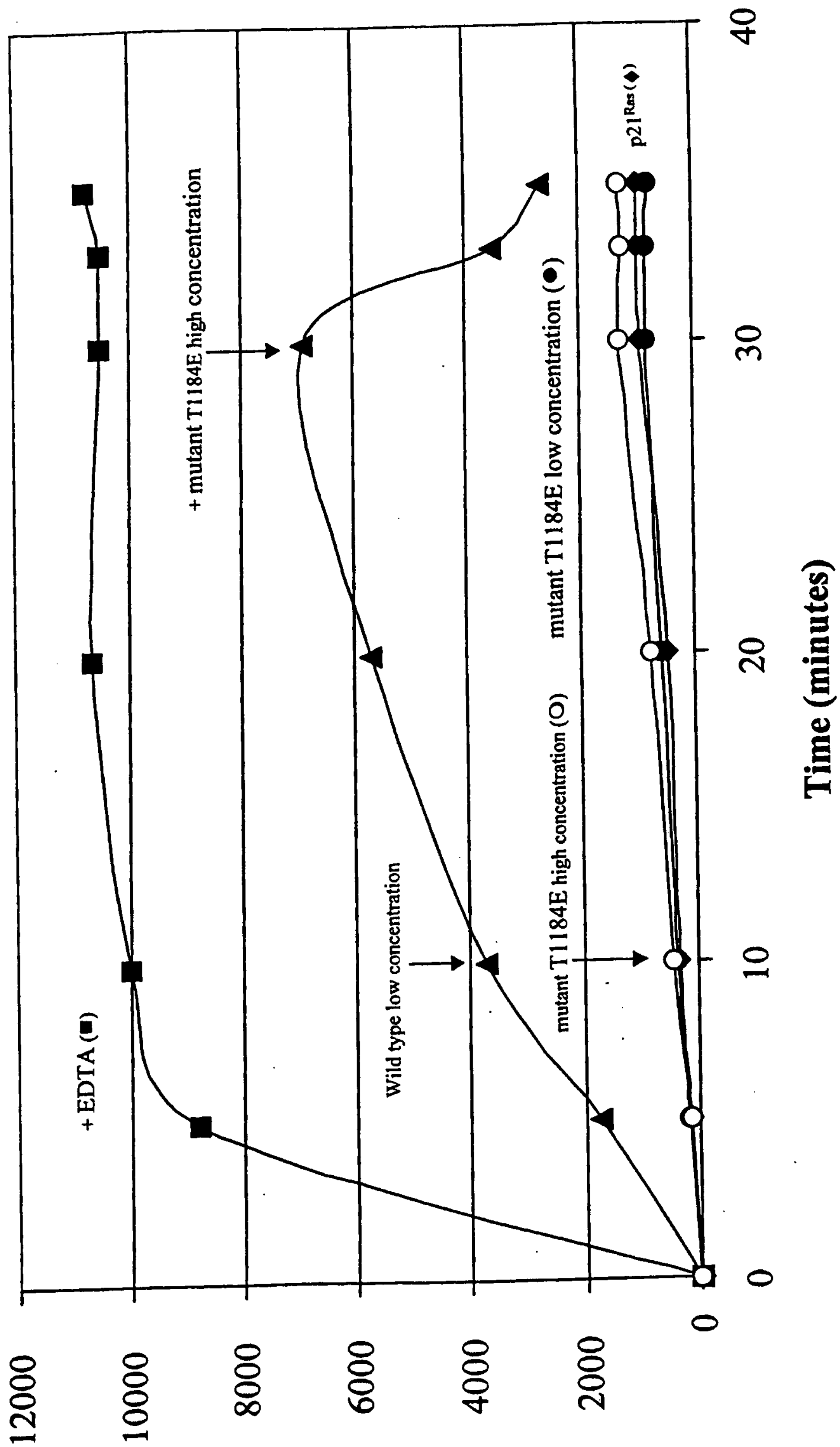
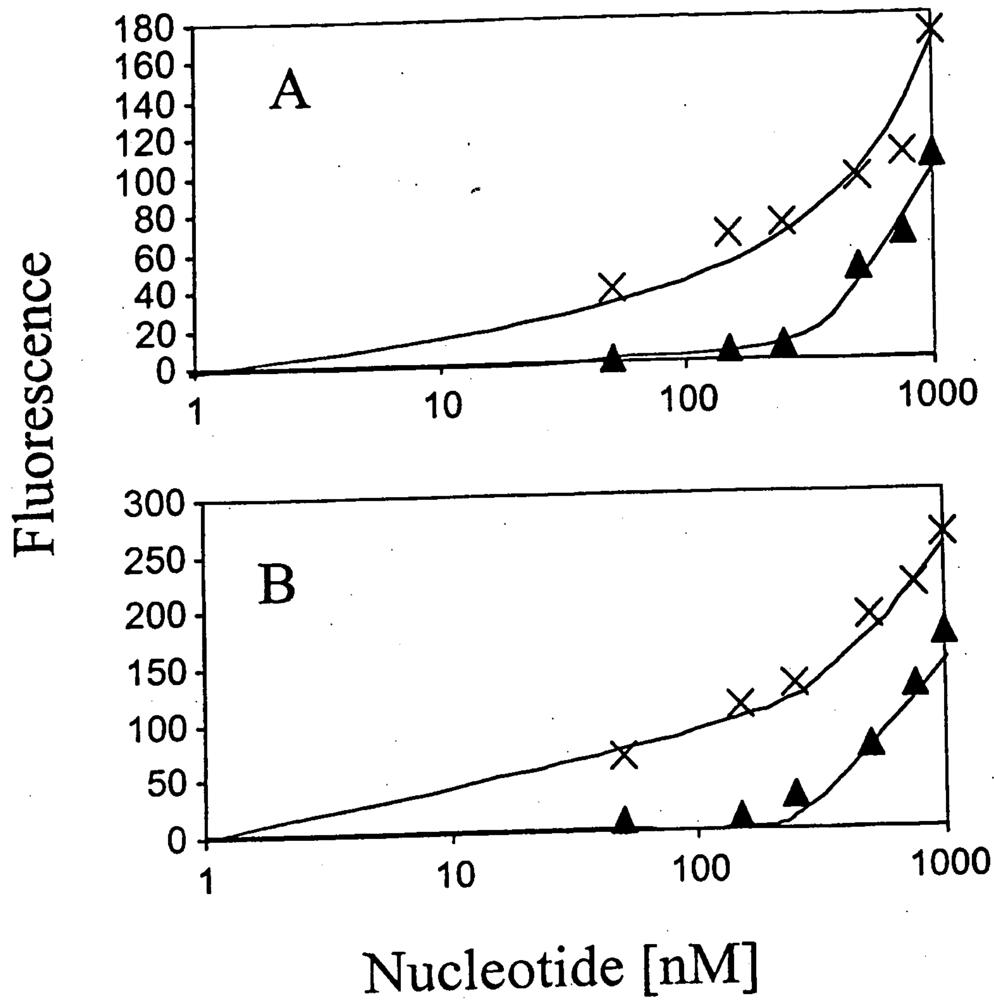

CDC25Mm
Sos
GNRP Catalytic Domain
Proline Rich Domain
PH Domain
DH Domain
IQ Domain

Figure 2

Figure 3



+ EDTA (■)
+ mutant T1184E high concentration
Wild type low concentration
mutant T1184E low concentration (●)
mutant T1184E high concentration (O)
p21Ras (◆)
12000
10000
8000
6000
4000
2000
0
0
10
20
30
40
Time (minutes)



A
B
180
160
140
120
100
80
60
40
20
0
300
250
200
150
100
50
0
1
10
100
1000
Fluorescence
Nucleotide [nM]

Figure 4

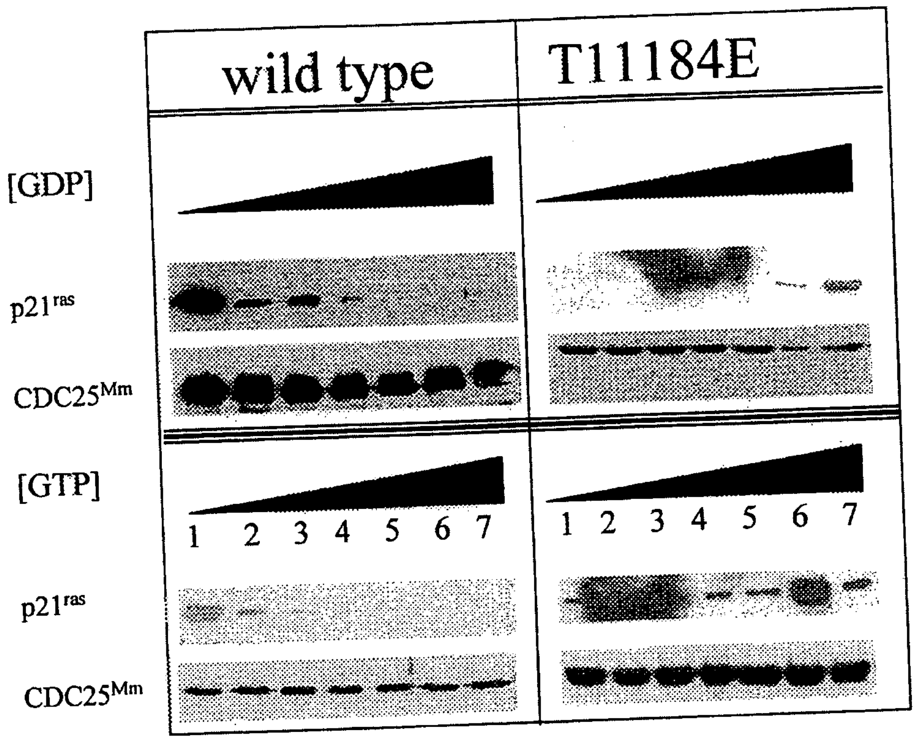
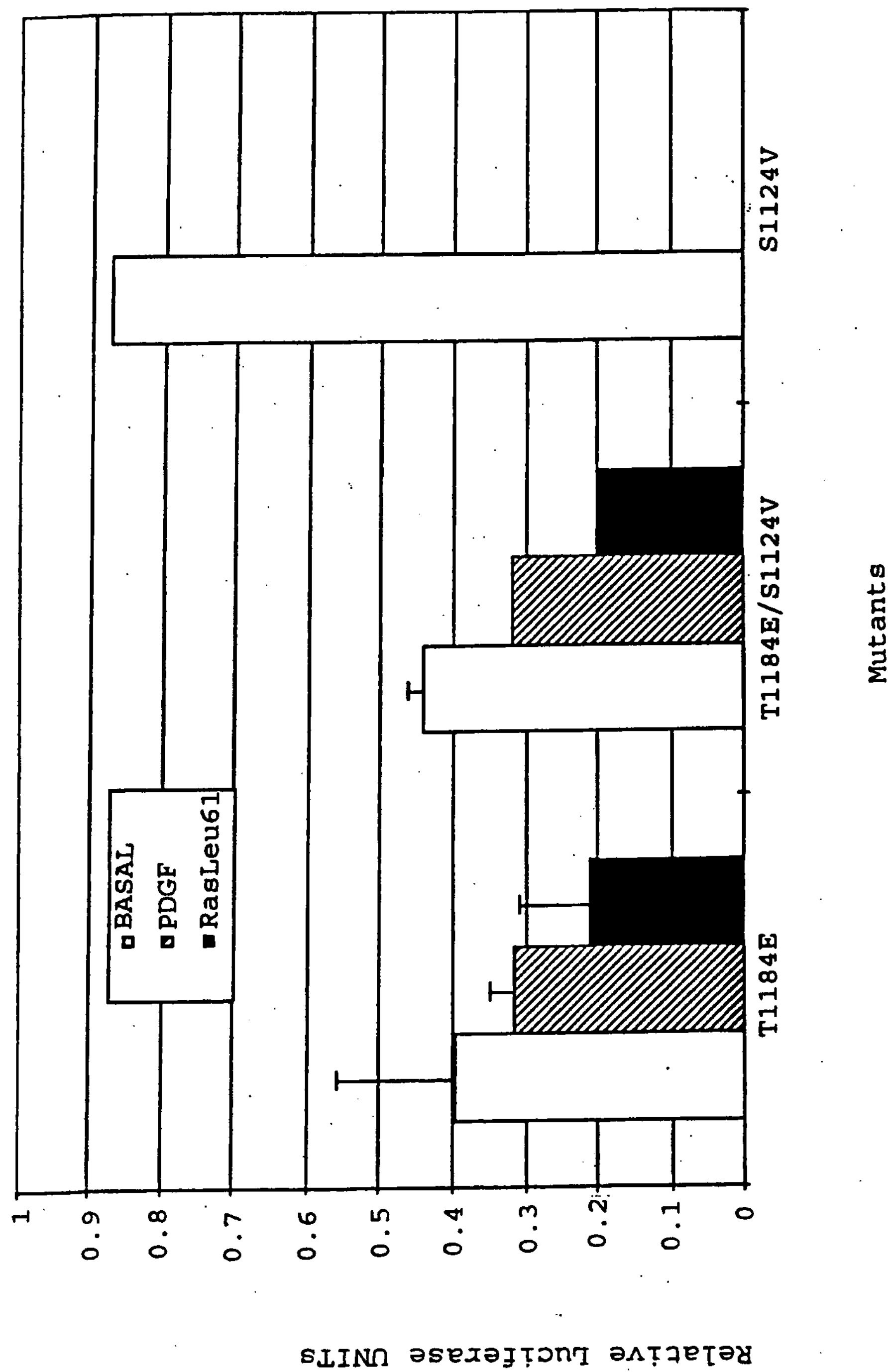

wild type
T11184E
[GDP]
p21ras
CDC25Mm
[GTP]
1 2 3 4 5 6 7
1 2 3 4 5 6 7
p21ras
CDC25Mm

Figure 5

Figure 6



BASAL
PDGF
RasLeu61
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
T1184E
T1184E/S1124V
S1124V
Mutants
Relative Luciferase UNITs

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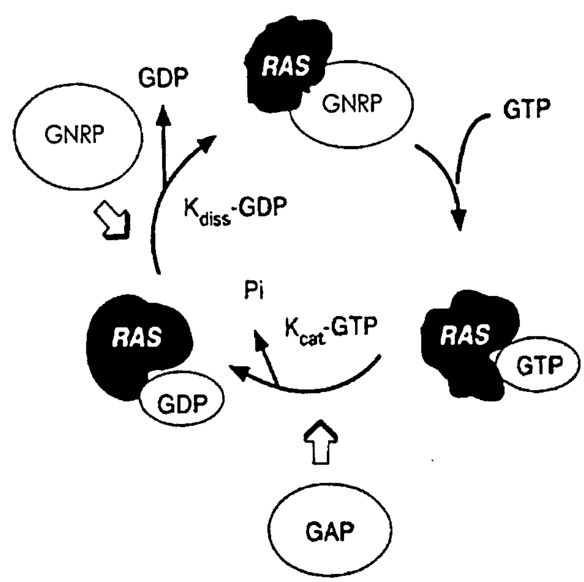
18 December 2003

[Continued on next page]

(54) Title: MUTANTS OF GNRPs AND VECTORS SUITABLE FOR THEIR EXPRESSION



(57) Abstract: The present invention relates to an amino acid sequence of a Guanine Nucleotide Releasing Protein (GNRP) spanning a portion of the catalytic domain in which threonine (T) corresponding to position 1184 of the protein of the GNRP class named CDC25^{Mm} is mutated to an acidic amino acid, the gene sequence encoding said amino acid sequence, and their application in the pharmaceutical field, in particular in the treatment of tumors, cardiovascular diseases, arterial restenosis and inflammatory states, or in the diagnostic field.

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

INTERNATIONAL SEARCH REPORT

International Application No
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A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/12 C07K14/47 C12N15/62 A61K38/16 G01N33/50
A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

BIOSIS, EPO-Internal, WPI Data, PAJ, STRAND, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	PARK W ET AL: "Identification of a dominant-negative mutation in the yeast CDC25 guanine nucleotide exchange factor for Ras" ONCOGENE,GB,BASINGSTOKE, HANTS, vol. 14, no. 7, 20 February 1997 (1997-02-20), pages 831-836, XP002098824 ISSN: 0950-9232 the whole document --- -/--	1-13,18

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 June 2000	19/07/2000
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Oderwald, H

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	CARRERA V ET AL.: "Mutations at position 1122 in the catalytic domain of the mouse ras-specific guanine nucleotide exchange factor CDC25Mm originate both loss-of-function and gain-of-function proteins" FEBS LETTERS, vol. 440, 1998, pages 291-296, XP002141331 the whole document ---	1-13,18
A	WO 93 21314 A (RHONE POULENC RORER SA ;SCHWEIGHOFFER FABIEN (FR); TOCQUE BRUNO (F) 28 October 1993 (1993-10-28) cited in the application the whole document ---	
A	PARK W ET AL: "Amino acid residues in the CDC25 guanine nucleotide exchange factor critical for interaction with Ras" MOLECULAR AND CELLULAR BIOLOGY,US,WASHINGTON, DC, vol. 14, no. 12, 12 December 1994 (1994-12-12), pages 8117-8122-8122, XP002098862 ISSN: 0270-7306 the whole document ---	
A	MARTEGANI E ET AL: "CLONING BY FUNCTIONAL COMPLEMENTATION OF A MOUSE CDNA ENCODING A HOMOLOGUE OF CDC25, A SACCHAROMYCES CEREVISIAE RAS ACTIVATOR" EMBO JOURNAL,GB,OXFORD UNIVERSITY PRESS, SURREY, vol. 11, no. 6, 1 June 1992 (1992-06-01), pages 2151-2157, XP000611568 ISSN: 0261-4189 cited in the application the whole document ---	
P,X	VANONI MARCO ET AL: "Characterization and properties of dominant-negative mutants of the ras-specific guanine nucleotide exchange factor CDC25Mm." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 274, no. 51, 17 December 1999 (1999-12-17), pages 36656-36662, XP002141332 ISSN: 0021-9258 the whole document -----	1-20

INTERNATIONAL SEARCH REPORT

national application No.
PCT/EP 00/00012

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Although claim 15 is directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

ormation on patent family members

International Application No
PCT/EP 00/00012

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9321314 A	28-10-1993	FR 2690162 A	22-10-1993
		CA 2131166 A	28-10-1993
		EP 0637334 A	08-02-1995
		JP 7505774 T	29-06-1995
		US 5656595 A	12-08-1997